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UNITED STATES PATENT APPLICATION

OF

Paul O. Sheppard, Scott R. Presnell

FOR

MAMMALIAN SECRETED PROTEINS

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Description

MAMMALIAN SECRETED PROTEINS

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This application is related to Provisional Application 60/215,446 filed on June 30, 2000. Under 35 U.S.C. § 119(e)(1), this application claims benefit of said Provisional Application.

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BACKGROUND OF THE INVENTION

Within the field of genetic engineering, polynucleotides encoding proteins of interest have been identified and cloned by methods that require a detailed knowledge of the structure and/or function of the polynucleotide or the encoded protein.

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These methods include hybridization screening, polymerase chain reaction (PCR), and expression cloning.

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With the more recent advent of large DNA sequence databases and the accompanying data analysis tools, identification of genes of interest is possible through the analysis of raw sequence data. Databases can be "mined" to locate sequences that resemble (are "homologous to") sequences of known function. Alignment of similar sequences can be used to place novel sequences within families of structurally similar sequences. These analytical tools can be combined with structural information obtained from, for example, X-ray crystallography to predict the higher order structure of a novel polypeptide. These analyses also facilitate prediction of polypeptide function. These recent technological advances have greatly increased the pace of gene discovery.

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Genetic engineering has made available a number of genes and proteins of pharmaceutical or other economic importance. Such proteins include, for example, tissue plasminogen activator (t-PA) (U.S. Patent No. 4,766,075), coagulation factor VII (U.S. Patent No. 4,784,950), erythropoietin (U.S. Patent No. 4,703,008), platelet derived growth factor (U.S. Patent No. 4,889,919), and various industrial enzymes (e.g., U.S. Patents Nos. 5,965,384; 5,942,431; and 5,922,586).

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Although estimates vary as to the amount of the human genome that has been identified to date, there remains a need in the art for further characterization of the human genome and the proteins encoded thereby. Previously unknown genes and proteins will be useful in the treatment and/or prevention of many human diseases, included diseases that have heretofore been refractory to treatment.

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SUMMARY OF THE INVENTION

Within one aspect of the invention there is provided an isolated polypeptide comprising fifteen contiguous amino acid residues of a polypeptide as shown in SEQ ID NO:M, wherein M is an even integer from 2 to 328. Within one
5 embodiment, the isolated polypeptide is from 15 to 723 amino acid residues in length. Within another embodiment, the at least fifteen contiguous amino acid residues of SEQ ID NO:M are operably linked via a peptide bond or polypeptide linker to a second polypeptide selected from the group consisting of maltose binding protein, an immunoglobulin constant region, a polyhistidine tag, and a peptide as shown in SEQ ID
10 NO:123. Within another embodiment, the polypeptide comprises at least 30 contiguous residues of SEQ ID NO:M. Within a further embodiment, the polypeptide comprises at least 47 contiguous residues of SEQ ID NO:M.

Within a second aspect of the invention there is provided an isolated, mature protein encoded by a sequence selected from the group consisting of SEQ ID
15 NO:N, wherein N is an odd integer from 1 to 327.

A third aspect of the invention provides isolated polynucleotides encoding the polypeptides disclosed above. Within certain embodiments of the invention the polynucleotides comprise a sequence of nucleotides as shown in SEQ ID NO:N, wherein N is an odd integer from 1 to 327.
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Within a fourth aspect of the invention there is provided an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide as shown in SEQ ID NO:M, wherein M is an even integer from 2 to 328; and a transcription terminator.

A fifth aspect of the invention provides a cultured cell comprising the
25 expression vector disclosed above. The cultured cell can be used, *inter alia*, within a method of producing a polypeptide, the method comprising (a) culturing the cell under conditions whereby the sequence of nucleotides is expressed, and (b) recovering the polypeptide. The invention also provides a polypeptide produced by this method.

Within a sixth aspect of the invention there is provided an isolated
30 polynucleotide encoding a fusion protein, wherein the fusion protein comprises a secretory peptide selected from the group consisting of secretory peptides shown in SEQ ID NO:M, wherein M is an even integer from 2 to 328, operably linked to a second polypeptide.

Within a seventh aspect of the invention there is provided an expression
35 vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a fusion protein as disclosed above; and a transcription terminator. The invention further provides a cultured cell comprising this expression

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vector, wherein the cell expresses the DNA segment and produces the encoded fusion protein. Also provided is a method of producing a protein comprising culturing the cell under conditions whereby the DNA segment is expressed, and recovering the second polypeptide. Within one embodiment the recovered second polypeptide is joined to a portion of a protein of SEQ ID NO: M, wherein M is an even integer from 2 to 328.

Within a further aspect of the invention there is provided a computer-readable medium encoded with a data structure comprising SEQ ID NO:X, wherein X is an integer from 1 to 328.

Within an additional aspect of the invention there is provided an antibody that specifically binds to a protein selected from of the group consisting of SEQ ID NO:M, wherein M is an even integer from 2 to 328.

Within an additional aspect, the invention provides an isolated polypeptide comprising fourteen contiguous amino acid residues of a polypeptide as shown in SEQ ID NO:M, wherein M is an even integer from 2 to 328. Within an embodiment, said isolated polypeptide comprising said isolated fourteen contiguous amino acid residues is selected from the polypeptides as shown in Table 1. Within another embodiment, said fourteen contiguous amino acid residues can be used in a fusion protein to facilitate the secretion of a second polypeptide of interest outside a cell.

Within an additional aspect the invention provides a method of detecting protein secretion from a cell or tissue comprising detecting a mature MSP selected from the group consisting of SEQ ID NO:M, wherein M is an even integer from 2 to 328 in conditioned media or membrane extracts.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci. USA* 82:7952-7954, 1985; see

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arranged in a way that does not exist naturally. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide or protein is substantially free of other polypeptides or proteins, particularly other polypeptides or proteins of animal origin. It is preferred to provide the polypeptides or proteins in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide or protein in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

A "mature protein" is a protein that is produced by cellular processing of a primary translation product of a DNA sequence. Such processing may include removal of a secretory signal peptide, sometimes in combination with a propeptide. Mature sequences can be predicted from full-length sequences using methods known in the art for predicting cleavage sites. See, for example, von Heijne (*Nuc. Acids Res.* 14:4683, 1986). The sequence of a mature protein can be determined experimentally by expressing a DNA sequence of interest in a eukaryotic host cell and determining the amino acid sequence of the final product. For proteins lacking secretory peptides, the primary translation product will be the mature protein.

"Operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator. When referring to polypeptides, "operably linked" includes both

covalently (e.g., by disulfide bonding) and non-covalently (e.g., by hydrogen bonding, hydrophobic interactions, or salt-bridge interactions) linked sequences, wherein the desired function(s) of the sequences are retained.

The term “ortholog” denotes a polypeptide or protein obtained from one 5 species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

“Paralogs” are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α -globin, β -globin, and myoglobin are paralogs of each other.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

30 A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as
35 carbohydrate groups are generally not specified, but may be present nonetheless.

A "secretory signal sequence" is a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs

the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

All references cited herein are incorporated by reference in their entirety.

5 The present invention is based in part upon the discovery of a group of novel, protein-encoding DNA molecules, designated "MSP" proteins, polypeptides and polynucleotides. These DNA molecules and the amino acid sequences that they encode are shown in SEQ ID NO:1 through SEQ ID NO:328. Sequence analysis predicts that each of the encoded proteins includes an amino-terminal secretory peptide. These
10 secretory peptides are shown below in Table 1, wherein residue numbers are in reference to the indicated SEQ ID NO. As will be understood by those skilled in the art, the cleavage sites predicted by conventional models of secretory peptide cleavage (e.g., von Heijne, *Nuc. Acids Res.* 14:4683, 1986) are not always exact and may vary by as much as ± 5 residues. In addition, cleavage may occur at multiple sites within 5
15 residues of the indicated position. The mature form of any given protein may thus consist of a plurality of species differing at their amino termini.

Table 1

<u>Protein</u>	<u>SEQ ID NO:</u>	<u>Residues 1-</u>
Z1055G2P	2	21
Z1183G7P	4	32
Z125G9P	6	17
Z128G9P	8	22
Z1297G2P	10	20
Z1345G3P	12	19
Z1345G5P	14	40
Z1659G4P	16	26
Z2007G2P	18	15
Z2263G5P	20	19
Z25829G3P	22	43
Z25999G1P	24	19
Z27842G1P	26	19
Z2812G3P	28	36
Z281G1P	30	45
Z2839G2P	32	48
Z287G9P	34	46
Z2887G7P	36	29
Z2972G5P	38	30
Z297G1P	40	38
Z3038G1P	42	25
Z321375G1P	44	36
Z3264G1P	46	14
Z3357G2P	48	16
Z4256G1P	50	17
Z4384G2P	52	17

Table 1 cont'd

Z446500G1P	54	19
Z4622G1P	56	22
Z4670G1P	58	31
Z555G20P	60	16
Z577G9P	62	20
Z700497G2P	64	25
Z700823G2P	66	31
Z700996G11P	68	23
Z701935G4P	70	36
Z702408G2P	72	20
Z74922G1P	74	21
Z790708G1P	76	34
Z791735G1P	78	19
Z792987G7P	80	28
Z793561G4P	82	16
Z794249G2P	84	20
Z799543G2P	86	14
Z807106G2P	88	22
Z807166G2P	90	15
Z807270G7P	92	15
Z807340G6P	94	28
Z807941G1P	96	31
Z809241G11P	98	14
Z809335G4P	100	21
Z809469G10P	102	22
Z835361G1P	104	32
Z835510G5P	106	20
Z835548G2P	108	32
Z835840G8P	110	18
Z835892G6P	112	26
Z835944G6P	114	21
Z835999G1P	116	14
Z836222G4P	118	16
Z836250G2P	120	17
Z836307G1P	122	15
Z836325G9P	124	17
Z836355G5P	126	22
Z837892G2P	128	33
Z838027G3P	130	28
Z838054G2P	132	16
Z838468G2P	134	33
Z842039G5P	136	18
Z842046G1P	138	50
Z846090G4P	140	21
Z846280G2P	142	27
Z846300G9P	144	23
Z846363G2P	146	33
Z846626G9P	148	24
Z846631G3P	150	15
Z849065G4P	152	25
Z849135G16P	154	40
Z849162G4P	156	14
Z849213G14P	158	14
Z849221G9P	160	20

Table 1 cont'd

Z849314G5P	162	23
Z849377G7P	164	18
Z849533G7P	166	21
Z849559G5P	168	22
Z849746G1P	170	19
Z849893G9P	172	16
Z855073G14P	174	21
Z855260G4P	176	15
Z869105G16P	178	14
Z869173G14P	180	20
Z869333G11P	182	21
Z869654G5P	184	30
Z869671G3P	186	15
Z869719G6P	188	16
Z873630G11P	190	16
Z873683G5P	192	30
Z873717G10P	194	22
Z873745G3P	196	31
Z873811G8P	198	31
Z873818G10P	200	26
Z874015G4P	202	35
Z874090G3P	204	20
Z874090G4P	206	20
Z874178G8P	208	18
Z874193G6P	210	26
Z875409G1P	212	14
Z877091G9P	214	24
Z877300G8P	216	33
Z877532G7P	218	17
Z877574G3P	220	41
Z877975G4P	222	41
Z887014G7P	224	18
Z887031G1P	226	18
Z887042G3P	228	25
Z887280G5P	230	22
Z887300G2P	232	27
Z888627G9P	234	24
Z888644G7P	236	21
Z888879G3P	238	16
Z890990G20P	240	21
Z891399G5P	242	18
Z891519G8P	244	23
Z891532G2P	246	14
Z891575G17P	248	15
Z891639G1P	250	40
Z891682G2P	252	20
Z891886G1P	254	18
Z892025G7P	256	34
Z892026G1P	258	14
Z903906G4P	260	25
Z908203G11P	262	16
Z908225G14P	264	31
Z908460G2P	266	24
Z908463G2P	268	19

Table 1 cont'd

Z908593G16P	270	18
Z908644G15P	272	20
Z908655G7P	274	14
Z908668G10P	276	18
Z908874G13P	278	23
Z908957G1P	280	15
Z909165G15P	282	38
Z909204G1P	284	22
Z909337G10P	286	29
Z909460G4P	288	17
Z910197G6P	290	18
Z912187G1P	292	24
Z913549G1P	294	42
Z930005G2P	296	19
Z930582G14P	298	25
Z930757G12P	300	27
Z930798G6P	302	25
Z930857G2P	304	30
Z930898G2P	306	20
Z931019G10P	308	17
Z931276G1P	310	15
Z931363G4P	312	23
Z931654G1P	314	18
Z931828G2P	316	18
Z931874G4P	318	29
Z935148G9P	320	21
Z935205G2P	322	24
Z935414G4P	324	18
Z935553G3P	326	26
Z935805G4P	328	33

A secretory peptide of a protein of the present invention can be used to direct the secretion of other proteins of interest from a host cell. Thus, the present invention provides, *inter alia*, fusions comprising such a secretory peptide of a protein disclosed herein operably linked to another protein of interest. The secretory peptide can be used to direct the secretion of other proteins of interest by joining a polynucleotide sequence encoding it, in the correct reading frame, to the 5' end of a sequence encoding the other protein of interest. Those skilled in the art will recognize that the resulting fused sequence may encode additional residues of a protein of the present invention at the amino terminus of the protein to be secreted. In the extreme case, the fusion may comprise an entire protein of the present invention fused to the amino terminus of a second protein, whereby secretion of the fusion protein is directed by the secretory peptide of the protein of the present invention. It will often be desirable to include a proteolytic cleavage site between the protein of the present invention (or portion thereof) and the other protein of interest. The joined polynucleotide sequences are then introduced into a host cell, which is cultured according to conventional

methods. The protein of interest is then recovered from the culture media. Methods for introducing DNA into host cells, culturing the cells, and isolating recombinant proteins are known in the art. Representative methods are summarized below.

- Higher order structures of the proteins of the present invention can be predicted by computer analysis using available software (e.g., the Insight II® viewer and homology modeling tools available from MSI, San Diego, CA; and King and Sternberg, *Protein Sci.* 5:2298-310, 1996). In addition, analytical algorithms permit the identification of homologies between newly discovered proteins and known proteins. Such homologies are indicative of related biological functions.
- Table 2 lists proteins described herein which have regions of similarity with other known proteins.

TABLE 2

PROTEIN	DESCRIPTION
Z1345G5P	SDC4_Human
Z2839G2P	Retrovirus unknown protein U5/1
Z2887G7P	PIR_T19298 <i>Caenorhabditis elegans</i> :
Z321375G1P	AK019752 <i>Mus musculus</i> adult male testis cDNA
Z807270G7P	U93565_1 Human L1 element L1.12 putative p150 gene
Z809241G11P	ALU1_Human ALU Subfamily J Sequence
Z835548G2P	AF026465_1 <i>Mus musculus</i> putative neuronal cell adhesion UH
Z835840G8P	FMOD_Human Fibromodulin
Z835944G6P	ID U93566_1 Human L1 element
Z838054G2P	SYT5_Human (membrane)
Z846363G2P	AK026318_1 <i>Homo sapiens</i> cDNA: FLJ22665 fis, HSIL8RB1 Human interleukin-8 receptor
Z849162G4P	PSP_G00866 Human secreted protein
Z869671G3P	PIR_T43481 probable mucin DKFZ
Z877574G3P	OTX1_Human Homeobox Protein OTX1
Z887300G2P	PSP_B07469 A human leucine-rich repeat protein
Z891519G8P	PIR_T38548 hypothetical zinc-finger protein
Z891886G1P	ER72_Human protein disulfide isomerase-related protein ERP-72 precursor
Z931874G4P	PIR_T19299 hypothetical protein C15A7.3 - <i>Caenorhabditis elegans</i>
Z935414G4P	MANB_BOVIN beta-mannosidase precursor

- Table 3 lists proteins described herein which have regions of identity with other known proteins.

TABLE 3

PROTEIN	DESCRIPTION
Z701935G4P	X86096_1 H.sapiens mRNA for E2F-4 protein
Z792987G7P	AB041736 Homo sapiens mRNA for SEZ6L
Z794249G2P	AL121756_2 Human DNA sequence from clone RP4-726C3
Z809469G10P	AB007925_1 Homo sapiens mRNA for KIAA045
Z835361G1P	BC005138 Homo sapiens, BUB3
Z835944G6P	U93566_1 Human L1 element
Z846300G9P	Human colon cancer cell line polynucleotide sequence
Z849065G4P	Polypeptide from WO200078961.50
Z849221G9P	AF253318_1 Homo sapiens GFR receptor alpha 4 protein
Z849314G5P	CLDI_Human
Z873630G11P	AB033072_1 Homo sapiens mRNA for KIAA1246 protein
Z887031G1P	AF119664_1 Homo sapiens transcriptional regulator
Z891639G1P	AK025362_1 Homo sapiens cDNA: FLJ21709 fis
Z903906G4P	M12987_6 F Plasmid DNA complete mini-F region
Z908203G11P	Human signal peptide containing protein HSPP-1
Z908874G13P	CLDI_Human Claudin-18
Z909337G10P	AR080331 Sequence 1 from patent US 5968768
Z930857G2P	Human prostacyclin-stimulating factor-2

A protein of the present invention can be prepared as a fusion protein by joining it to a second polypeptide or a plurality of additional polypeptides. Suitable second polypeptides include amino- or carboxyl-terminal extensions, such as linker peptides of up to about 20-25 residues and extensions that facilitate purification (affinity tags) as disclosed above. A protein of interest can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Preferred dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin-polypeptide fusions can be expressed in genetically engineered cells to produce a variety of multimeric analogs of a protein of interest. Fusion proteins can also comprise auxiliary domains that target the protein of interest to specific cells, tissues, or macromolecules (e.g., collagen). For example, a protein of interest can be targeted to a predetermined cell type by fusing it to a ligand that specifically binds to a receptor on the surface of a target cell. In this way, proteins can be targeted for therapeutic or diagnostic purposes. A protein can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Protein fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., *Connective Tissue Research* 34:1-9, 1996. Proteins of the present invention can also be used as targeting moieties within fusion proteins comprising, for example, cytokines, cytotoxins, or other biologically active polypeptide moieties.

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Protein fusions of the present invention will usually contain not more than about 1,200 amino acid residues joined to the MSP protein. For example, an MSP protein can be fused to *E. coli* β -galactosidase (1,021 residues; see Casadaban et al., *J. Bacteriol.* 143:971-980, 1980), a 10-residue spacer, and a 4-residue factor Xa cleavage site. Such a protein comprising, for example, MSPZ809335G4P (SEQ ID NO:100), contains 723 amino acid residues. In a second example, an MSP protein can be fused to maltose binding protein (approximately 370 residues), a 4-residue cleavage site, and a 6-residue polyhistidine tag.

As disclosed above, the proteins of the present invention or portions thereof can also be used to direct the secretion of a second protein. When such fusions are designed so that the secreted protein retains a portion of the protein of the present invention, the fusion protein can be purified by means that exploit the properties of the protein of the present invention. Typical of such methods is immunoaffinity chromatography using an antibody directed against a protein of the present invention. When such a fusion is engineered to contain a cleavage site at the fusion point, the fusion can be cleaved and the protein of interest recovered free of extraneous sequence.

As secreted proteins, MSP can be useful to monitor secretion of proteins in general from cells or tissue. In doing so, cell lines or tissues shown to express the proteins of the present invention can be monitored to detect for example if the cell or tissue has a defective secretory pathway. Thus, cell membrane extracts or conditioned media from the cell or tissue can be tested for the presence or absence of the mature MSP protein. Similarly, when trying to identify if a recombinantly expressed protein is has a functional signal peptide, the molecules of the present invention can be used as a positive control.

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the proteins disclosed above. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. The amino acid sequence information provided herein can be used by one of ordinary skill in the art to generate degenerate sequences comprising all nucleotide sequences encoding a particular polypeptide. Table 4 sets forth the one-letter codes used to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

TABLE 5

Amino Acid	One-Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	CAN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAV
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN
Gap	-	---	

- One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequences may encode variant amino acid sequences,

Methods for preparing DNA and RNA are well known in the art.

tissue or cell that produces large amounts of the cognate mRNA. Such tissues and cells

(Thomas, *Proc. Natl. Acad. Sci. USA* 77:5201, 1980). Databases of expressed sequence

assigned to specific cell or tissue sources on the basis of their abundance within

A panel of cDNAs from human tissues can be screened for tissue

available first strand cDNA (Clontech laboratories, Inc. Palo Alto CA) and would

panel can be set up in a 96-well format that further included a human genomic DNA

well as a negative control sample. Each well can contain approximately 0.2–100 ng/ul

oligonucleotide primers. DNA polymerase (Ex Taq™, TAKARA Shuzo Co. Ltd.

Laboratories, Inc.) with the appropriate supplied buffer, dNTP mix (TAKARA Shuzo

Genetics, Inc., Huntsville, AL) to each sample on the panel. The amplification of the

for 30 seconds, 60°C for 20 seconds, and 72°C for 30 seconds; followed by incubation at

agarose gel electrophoresis using a 4% agarose gel. The results of a PCR that was set

σ_T is the standard deviation shown in Table 6.

Table 6

Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Z909460G4P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	y
Z931828G2p	n	n	y	y	y	y	y	n	y	n	n	y	y	y	n	n	n	n	y	n	y	y
Z1055G2P	n	n	n	n	n	n	y	n	n	y	y	y	y	y	n	y	y	y	y	y	n	y
Z1183G7P	y	y	y	n	y	y	y	y	n	n	n	n	n	n	n	n	n	n	n	y	n	y
Z1345G3P	y	n	y	n	n	y	n	n	n	n	n	n	n	n	y	n	y	y	y	y	n	y
Z1659G4P	n	y	y	n	y	y	n	n	n	n	n	n	n	n	n	n	n	n	n	y	n	y
Z2007G2P	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	y	n	y
Z2599G1P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	y
Z281G1P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z4622G1P	y	y	y	y	y	y	y	n	y	y	y	y	y	y	y	y	y	y	y	y	n	y
Z4670G1P	n	n	n	y	n	y	n	n	y	y	y	y	n	y	y	n	y	y	y	y	n	y
Z555G20P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	y
Z577G9P	y	n	y	y	n	y	n	n	y	n	y	y	y	y	y	y	y	y	y	y	n	y
Z702408G2P	n	n	n	y	nd	nd	n	n	n	n	n	n	n	n	n	n	n	n	n	n	nd	n
Z74922G1P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z793561G4P	n	n	n	n	n	n	n	n	y	n	y	n	n	y	nd	y	y	n	y	y	n	y
Z799543G2P	n	n	y	n	y	n	n	n	y	n	y	n	y	n	nd	n	y	y	n	n	n	y
Z807166G2P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	y
Z835892G6P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	nd	n	n	n	n	n	n	y
Z846222G4P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z836355G5P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z837892G2P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z842046G1P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	y
Z846626G9P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	nd	n	n	n	n	n	n	y
Z846631G3P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z849213G14P	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Z849746G1P	y	n	n	n	n	n	n	n	n	n	n	y	y	n	nd	n	n	n	n	n	y	N

Table 6 cont.

[illegible]

Table 6 cont.

[illegible]

Table 6 cont.

[illegible]

Tissues screened were: 1, brain; 2, heart; 3, kidney; 4, liver; 5, lung; 6, pancreas; 7, placenta; 8, skeletal muscle; 9, colon; 10, ovary; 11, peripheral blood leukocytes; 12, prostate; 13, small intestine; 14, spleen; 15, testis; 16, thymus; 17, bone marrow; 18, fetal liver; 19, lymph node; 20, tonsil; 21, H₂O; 22, genomic DNA. Y= yes; n=no; nd=not determined.

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Probes and primers as disclosed herein can be used for cloning allelic, orthologous, and paralogous sequences. Allelic variants of the disclosed sequences can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Orthologous sequences can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the protein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned by PCR using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to the encoded protein. Similar techniques can also be applied to the isolation of genomic clones. Orthologous and paralogous sequences can be identified from libraries by probing blots at low stringency and washing the blots at successively higher stringency until background is suitably reduced.

Probes and primers disclosed herein can be used to clone 5' non-coding regions of a corresponding gene. Such promoter elements can thus be used to direct the tissue-specific expression of heterologous genes in, for example, transgenic animals or patients treated with gene therapy. Cloning of 5' flanking sequences also facilitates production of a protein of interest by "gene activation" as disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous gene in a cell is altered by introducing into its locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a 5' non-coding sequence that permits homologous recombination of the construct with the endogenous locus, whereby the sequences within the construct become operably linked with the endogenous coding sequence. In this way, an endogenous promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

The polynucleotides of the present invention further include polynucleotides encoding the fusion proteins, including signal peptide fusions, disclosed above.

In studying cell biology it is useful to monitor changing levels of mRNA as a function of cell and tissue development. As a set of cDNA molecules, the polynucleotides of the present invention, for example SEQ ID NO:N, wherein N is an

odd integer from 1 to 327, can also be used to screen for levels of mRNA of the polypeptides, i.e., as shown in SEQ ID NO:M, wherein M is an even integer from 2 to 328. Screening for message level fluctuations of the molecules of the present invention are useful for studying, for example, cell replication, activation, and quiescence.

The present invention further provides a computer-readable medium encoded with a data structure that provides at least one of SEQ ID NO:1 through SEQ ID NO:328. Suitable forms of computer-readable media include magnetic media and optically-readable media. Examples of magnetic media include a hard or fixed drive, a random access memory (RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP® disk. Optically readable media are exemplified by compact discs (e.g., CD-read only memory (ROM), CD-rewritable (RW), and CD-recordable), and digital versatile/video discs (DVD) (e.g., DVD-ROM, DVD-RAM, and DVD+RW).

The polypeptides of the present invention, including full-length proteins, biologically active fragments, immunogenic fragments, and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are generally preferred for the production of proteins having higher eukaryotic-type post-translational modifications (e.g., γ -carboxylation) and for making proteins, especially secretory proteins, for pharmaceutical use in humans. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., eds., *Current Protocols in Molecular Biology*, Green and Wiley and Sons, NY, 1993.

In general, a DNA sequence encoding a polypeptide of interest is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers can be provided on separate vectors, and replication of the exogenous DNA can be achieved through integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the protein of interest, or may be derived from another secreted protein (e.g., t-PA; see U.S. Patent No. 5,641,655) or synthesized *de novo*. The secretory signal sequence is operably linked to the DNA sequence encoding the protein of interest, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized protein into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the protein of interest, although certain secretory signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells are suitable hosts for use within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann et al., *EMBO J.* 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., *ibid.*), and liposome-mediated transfection (Hawley-Nelson et al., *Focus* 15:73, 1993; Ciccarone et al., *Focus* 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed by, for example, Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Manassas, Virginia. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter. Within an alternative embodiment, adenovirus vectors can be employed. See, for example, Garnier et al., *Cytotechnol.* 15:145-55, 1994.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and

are able to pass the gene of interest to their progeny are referred to as "stable transfectants." An exemplary selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. An exemplary amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV). See, King and Possee, The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and Richardson, Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Humana Press, Totowa, NJ, 1995. Recombinant baculovirus can also be produced through the use of a transposon-based system described by Luckow et al. (*J. Virol.* 67:4566-4579, 1993). This system, which utilizes transfer vectors, is commercially available in kit form (Bac-to-Bac™ kit; Life Technologies, Rockville, MD). See also, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971-976, 1990; Bonning et al., *J. Gen. Virol.* 75:1551-1556, 1994; and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543-1549, 1995.

For protein production, the recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda* (e.g., Sf9 or Sf21 cells) or *Trichoplusia ni* (e.g., High Five™ cells; Invitrogen, Carlsbad, CA). See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. See also, U.S. Patent No. 5,300,435. Serum-free media are used to grow and maintain the cells. Suitable media formulations are known in the art and can be obtained from commercial suppliers. The cells are grown up from an inoculation density of approximately 2-5 x 10⁵ cells to a density of 1-2 x 10⁶ cells, at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally described in available laboratory manuals (e.g., King and Possee, *ibid.*; O'Reilly et al., *ibid.*; Richardson, *ibid.*). See also, Guarino et al., U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454.

Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., *J. Gen. Microbiol.* 132:3459-3465, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533. Production of recombinant proteins in *Pichia methanolica* is disclosed in U.S. Patents No. 5,716,808, 5,736,383, 5,854,039, and 5,888,768; and WIPO publications WO 99/14347 and WO 99/14320.

Other higher eukaryotic cells, including plant cells and avian cells, can also be used as hosts according to methods commonly known in the art. For example, the use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., *J. Biosci. (Bangalore)* 11:47-58, 1987.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., *ibid.*). When expressing a polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the

cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

It is preferred to purify the polypeptides and proteins of the present invention to $\geq 80\%$ purity, more preferably to $\geq 90\%$ purity, even more preferably $\geq 95\%$ purity, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified polypeptide or protein is substantially free of other polypeptides or proteins, particularly those of animal origin.

Expressed recombinant proteins (including single polypeptide chains, chimeric polypeptides, and polypeptide multimers) are purified by conventional protein purification methods, typically by a combination of chromatographic techniques. See, in general, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988; and Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York, 1994. Proteins comprising a polyhistidine affinity tag (typically about 6 histidine residues) are purified by affinity chromatography on a nickel chelate resin. See, for example, Houchuli et al., *BioTechnol.* 6: 1321-1325, 1988. Proteins comprising a glu-glu tag can be purified by immunoaffinity chromatography essentially as disclosed by Grussenmeyer et al., *ibid.*

Proteins of the present invention and fragments thereof can also be prepared through chemical synthesis according to methods known in the art, including exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. See, for example, Merrifield, *J. Am. Chem. Soc.* **85**:2149, 1963; Stewart et al., Solid Phase Peptide Synthesis (2nd edition), Pierce Chemical Co., Rockford, IL, 1984; Bayer and Rapp, *Chem. Pept. Prot.* **3**:3, 1986; and Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford, 1989.

Biological activities of the proteins of the present invention can be measured *in vitro* using cultured cells or *in vivo* by administering molecules of the claimed invention to the appropriate animal model. Many such assays and models are known in the art. Guidance in initial assay selection is provided by structural predictions and sequence alignments. However, even if no functional prediction is made, the activity of a protein can be elucidated by known methods, including, for example, screening a variety of target cells for a biological response, other *in vitro* assays, expression in a host animal, or through the use of transgenic and/or “knockout” animals. Through the application of robotics, many *in vitro* assays can be adapted to rapid, high-throughput screening of a large number of samples. Target cells for use in activity assays include, without limitation, vascular cells (especially endothelial cells and smooth muscle cells), hematopoietic (myeloid and lymphoid) cells, liver cells (including hepatocytes, fenestrated endothelial cells, Kupffer cells, and Ito cells), fibroblasts (including human dermal fibroblasts and lung fibroblasts), neurite cells (including astrocytes, glial cells, dendritic cells, and PC-12 cells), fetal lung cells, articular synoviocytes, pericytes, chondrocytes, osteoblasts, adipocytes, and prostate epithelial cells. Endothelial cells and hematopoietic cells are derived from a common ancestral cell, the hemangioblast (Choi et al., *Development* 125:725-732, 1998).

Biological activity can be measured with a silicon-based biosensor microphysiometer that measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent physiologic cellular responses. An exemplary such device is the Cytosensor™ Microphysiometer manufactured by Molecular Devices, Sunnyvale, CA. A variety of cellular responses, such as cell proliferation, ion transport, energy production, inflammatory response, regulatory and

receptor activation, and the like, can be measured by this method. See, for example, McConnell et al., *Science* 257:1906-1912, 1992; Pitchford et al., *Meth. Enzymol.* 228:84-108, 1997; Arimilli et al., *J. Immunol. Meth.* 212:49-59, 1998; and Van Liefde et al., *Eur. J. Pharmacol.* 346:87-95, 1998. The microphysiometer can be used for

- 5 assaying adherent or non-adherent eukaryotic or prokaryotic cells. By measuring extracellular acidification changes in cell media over time, the microphysiometer directly measures cellular responses to various stimuli, including agonistic and antagonistic stimuli. Preferably, the microphysiometer is used to measure responses of a eukaryotic cell known to be responsive to the protein of interest, compared to a
- 10 control eukaryotic cell that does not respond to the protein of interest. Responsive eukaryotic cells comprise cells into which a receptor for the protein of interest has been transfected, as well as naturally responsive cells. Differences in the response of cells exposed to the protein of interest, relative to a control not so exposed, are a direct measurement of protein-modulated cellular responses. Such responses can be assayed
- 15 under a variety of stimuli. The present invention thus provides methods of identifying agonists and antagonists of proteins of interest, comprising providing cells responsive to a selected protein, culturing a first portion of the cells in the absence of a test compound, culturing a second portion of the cells in the presence of a test compound, and detecting a change in a cellular response of the second portion of the cells as
- 20 compared to the first portion of the cells. The change in cellular response is shown as a measurable change in extracellular acidification rate. Culturing a third portion of the cells in the presence of the protein of interest and the absence of a test compound provides a positive control and a control to compare the agonist activity of a test compound with that of the protein of interest. Antagonists can be identified by
- 25 exposing the cells to the protein of interest in the presence and absence of the test compound, whereby a reduction in protein-stimulated activity is indicative of antagonist activity in the test compound.

- As expressed secreted proteins the MSP polypeptides disclosed herein can be used to screen for cell metabolism effecting receptors. Thus, the polypeptides of
- 30 the present invention are useful for identifying new target receptors and drug design. A subset of the polypeptides disclosed herein can include membrane-bound receptors. This subset can include amongst other receptors, homologs of G protein coupled receptors, which are important mediators for cell activities including the synthesis of second messengers. Thus a set of these G protein couple receptors can mediate cel
 - 35 differentiation and proliferation, or hormone expression *in vitro* and *in vivo*.

Assays measuring cell proliferation or differentiation are well known in the art. For example, assays measuring proliferation include such assays as

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Cell migration is assayed essentially as disclosed by Kähler et al. (*Arteriosclerosis, Thrombosis, and Vascular Biology* 17:932-939, 1997). A protein is

considered to be chemotactic if it induces migration of cells from an area of low protein concentration to an area of high protein concentration. A typical assay is performed using modified Boyden chambers with a polystyrene membrane separating the two chambers (Transwell; Corning Costar Corp.). The test sample, diluted in medium containing 1% BSA, is added to the lower chamber of a 24-well plate containing Transwells. Cells are then placed on the Transwell insert that has been pretreated with 0.2% gelatin. Cell migration is measured after 4 hours of incubation at 37°C. Non-migrating cells are wiped off the top of the Transwell membrane, and cells attached to the lower face of the membrane are fixed and stained with 0.1% crystal violet. Stained cells are then extracted with 10% acetic acid and absorbance is measured at 600 nm. Migration is then calculated from a standard calibration curve. Cell migration can also be measured using the matrigel method of Grant et al. ("Angiogenesis as a component of epithelial-mesenchymal interactions" in Goldberg and Rosen, *Epithelial-Mesenchymal Interaction in Cancer*, Birkhäuser Verlag, 1995, 235-248; Baatout, *Anticancer Research* 17:451-456, 1997).

Proteins can be assayed for the ability to modulate axon guidance and growth. Suitable assays that detect changes in neuron growth patterns include, for example, those disclosed in Hastings, WIPO Publication WO 97/29189 and Walter et al., *Development* 101:685-96, 1987. Assays to measure the effects on neuron growth are well known in the art. For example, the C assay (e.g., Raper and Kapfhammer, *Neuron* 4:21-9, 1990 and Luo et al., *Cell* 75:217-27, 1993) can be used to determine collapsing activity of a protein of interest on growing neurons. Other methods that can assess protein-induced inhibition of neurite extension or divert such extension are also known. See, Goodman, *Annu. Rev. Neurosci.* 19:341-77, 1996. Conditioned media from cells expressing a protein of interest, or aggregates of such cells, can be placed in a gel matrix near suitable neural cells, such as dorsal root ganglia (DRG) or sympathetic ganglia explants, which have been co-cultured with nerve growth factor. Compared to control cells, protein-induced changes in neuron growth can be measured (as disclosed by, for example, Messersmith et al., *Neuron* 14:949-59, 1995 and Puschel et al., *Neuron* 14:941-8, 1995). Neurite outgrowth can be measured using neuronal cell suspensions grown in the presence of molecules of the present invention. See, for example, O'Shea et al., *Neuron* 7:231-7, 1991 and DeFreitas et al., *Neuron* 15:333-43, 1995.

Cell adhesion activity is assayed essentially as disclosed by LaFleur et al. (*J. Biol. Chem.* 272:32798-32803, 1997). Briefly, microtiter plates are coated with the test protein, non-specific sites are blocked with BSA, and cells (such as smooth muscle cells, leukocytes, or endothelial cells) are plated at a density of approximately

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Receptor binding can be measured by the competition binding method of Labriola-Tompkins et al., *Proc. Natl. Acad. Sci. USA* 88:11182-11186, 1991. In an exemplary assay for IL-1 receptor binding, membranes prepared from EL-4 thymoma cells (Paganelli et al., *J. Immunol.* 138:2249-2253, 1987) are incubated in the presence

IL-1 for binding to cultured human dermal fibroblasts is measured according to the method of Dower et al. (*Nature* **324**:266-268, 1986). Briefly, cells are incubated in a round-bottomed, 96-well plate in a suitable culture medium (e.g., RPMI 1640 containing 1% BSA, 0.1% Na azide, and 20 mM HEPES pH 7.4) at 8°C on a rocker platform in the presence of labeled IL-1. Various concentrations of test protein are added. After the incubation (typically about two hours), cells are separated from unbound label by centrifuging 60-µl aliquots through 200 µl of phthalate oils in 400-µl polyethylene centrifuge tubes and excising the tips of the tubes with a razor blade as disclosed by Segal and Hurwitz, *J. Immunol.* **118**:1338-1347, 1977. Receptor binding assays for other cell types are known in the art. See, for example, Bowen-Pope and Ross, *Methods Enzymol.* 109:69-100, 1985.

Receptor binding can also be measured using immobilized receptors or ligand-binding receptor fragments. For example, an immobilized receptor can be exposed to its labeled ligand and unlabeled test protein, whereby a reduction in labeled ligand binding compared to a control is indicative of receptor-binding activity in the test protein. Within another format, a receptor or ligand-binding receptor fragment is immobilized on a biosensor (e.g., BIAcore™, Pharmacia Biosensor, Piscataway, NJ) and binding is determined. Antagonists of the native ligand will exhibit receptor binding but will exhibit essentially no activity in appropriate activity assays or will reduce the ligand-mediated response when combined with the native ligand. In view of the low level of receptor occupancy required to produce a response to some ligands (e.g., IL-1), a large excess of antagonist (typically a 10- to 1000-fold molar excess) may be necessary to neutralize ligand activity.

Receptor activation can be detected in target cells by: (1) measurement of adenylate cyclase activity (Salomon et al., *Anal. Biochem.* **58**:541-48, 1974; Alvarez and Daniels, *Anal. Biochem.* **187**:98-103, 1990); (2) measurement of change in intracellular cAMP levels using conventional radioimmunoassay methods (Steiner et al., *J. Biol. Chem.* **247**:1106-13, 1972; Harper and Brooker, *J. Cyclic Nucl. Res.* **1**:207-18, 1975); or (3) through use of a cAMP scintillation proximity assay (SPA) method (such as available from Amersham Corp., Arlington Heights, IL).

Proteins can be tested for serine protease activity or proteinase inhibitory activity using conventional assays. Substrate cleavage is conveniently assayed using a tetrapeptide that mimics the cleavage site of the natural substrate and which is linked,

- via a peptide bond, to a carboxyl-terminal para-nitro-anilide (pNA) group. The protease hydrolyzes the bond between the fourth amino acid residue and the pNA group, causing the pNA group to undergo a dramatic increase in absorbance at 405 nm. Suitable substrates can be synthesized according to known methods or obtained from commercial suppliers. Inhibitory activity is measured by adding a test sample to a reaction mixture containing enzyme and substrate, and comparing the observed enzyme activity to a control (without the test sample). A variety of such assays are known in the art, including assays measuring inhibition of trypsin, chymotrypsin, plasmin, cathepsin G, and human leukocyte elastase. See, for example, Petersen et al., *Eur. J. Biochem.* 235:310-316, 1996. In a typical procedure, the inhibitory activity of a test compound is measured by incubating the test compound with the proteinase, then adding an appropriate substrate, typically a chromogenic peptide substrate. See, for example, Norris et al. (*Biol. Chem. Hoppe-Seyler* 371:37-42, 1990). Various concentrations of the inhibitor are incubated in the presence of trypsin, plasmin, and plasma kallikrein in a low-salt buffer at pH 7.4, 25°C. After 30 minutes, the residual enzymatic activity is measured by the addition of a chromogenic substrate (e.g., S2251 (D-Val-Leu-Lys-Nan) or S2302 (D-Pro-Phe-Arg-Nan), available from Kabi, Stockholm, Sweden) and a 30-minute incubation. Inhibition of enzyme activity is indicated by a decrease in absorbance at 405 nm or fluorescence Em at 460 nm. From the results, the apparent inhibition constant K_i is calculated. When a serine protease is prepared as an active precursor (e.g., comprising N-terminal residues 1-109 of SEQ ID NO:2), it is activated by cleavage with a suitable protease (e.g., furin (Steiner et al., *J. Biol. Chem.* 267:23435-23438, 1992)) prior to assay. Assays of this type are well known in the art. See, for example, Lottenberg et al., *Thrombosis Research* 28:313-332, 1982; Cho et al., *Biochem.* 23:644-650, 1984; Foster et al., *Biochem.* 26:7003-7011, 1987). The inhibition of coagulation factors (e.g., factor VIIa, factor Xa) can be measured using chromogenic substrates or in conventional coagulation assays (e.g., clotting time of normal human plasma; Dennis et al., *J. Biol. Chem.* 270:25411-25417, 1995).
- Blood coagulation and chromogenic assays, which can be used to detect both procoagulant, anticoagulant, and thrombolytic activities, are known in the art. For example, pro- and anticoagulant activities can be measured in a one-stage clotting assay using platelet-poor or factor-deficient plasma (Levy and Edgington, *J. Exp. Med.* 151:1232-1243, 1980; Schwartz et al., *J. Clin. Invest.* 67:1650-1658, 1981). As disclosed by Anderson et al. (*Proc. Natl. Acad. Sci. USA* 96:11189-11193, 1999), the effect of a test compound on platelet activation can be determined by a change in turbidity, and the procoagulant activity of activated platelets can be determined in a

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Anti-inflammatory activity can be tested in animal models of inflammatory disease. For example, animal models of psoriasis include the analysis of histological alterations in adult mouse tail epidermis (Hofbauer et al, *Brit. J. Dermatol.* 118:85-89, 1988; Bladon et al., *Arch Dermatol. Res.* 277:121-125, 1985). In this

model, anti-psoriatic activity is indicated by the induction of a granular layer and orthokeratosis in areas of scale between the hinges of the tail epidermis. Typically, a

topical ointment comprising a test compound is applied daily for seven consecutive days, then the animal is sacrificed, and tail skin is examined histologically. An

additional model is provided by grafting psoriatic human skin to congenitally athymic (nude) mice (Krueger et al., *J. Invest. Dermatol.* 64:307-312, 1975). Such grafts have

been shown to retain the characteristic histology for up to eleven weeks. As in the mouse tail model, the test composition is applied to the skin at predetermined intervals

for a period of one to several weeks, at which time the animals are sacrificed and the skin grafts examined histologically. A third model has been disclosed by Fretland et al. (J. Neurosurg. 14:737-739, 1980). Briefly, inflammation is induced in mice by

epidermis by topically applying phorbol ester (phorbol-12-myristate-13-acetate; PMA), typically at ca. 2 g/ml in acetone, to one ear and vehicle to the contralateral ear. Test

compounds are applied concurrently with the PMA, or may be given orally. Histological analysis is performed at 96 hours after application of PMA. This model

duplicates many symptoms of human psoriasis, including edema, inflammatory cell diapedesis and infiltration, high LTB₄ levels and epidermal proliferation.

Cerebral ischemia can be studied in a rat model as disclosed by Relton et al. (*ibid.*) and Loddick et al. (*ibid.*).

The effect of a test protein on primordial endothelial cells in angiogenesis can be assayed in the chick chorioallantoic membrane angiogenesis assay

(Leung, *Science* 246:1306-1309, 1989; Ferrara, *Ann. NY Acad. Sci.* 752:246-256, 1995). Briefly, a small window is cut into the shell of an eight-day old fertilized egg,

and a test substance is applied to the chorioallantoic membrane. After 72 hours, the membrane is examined for neovascularization. Embryo microinjection of early stage

quail (*Coturnix coturnix japonica*) embryos can also be used (Drake et al., *Proc. Natl. Acad. Sci. USA* **92**:7657-7661, 1995). Briefly, a solution containing the protein is

of early-stage embryos using a micropipette and micromanipulator system. After injection, embryos are placed ventral side down on a nutrient agar medium and

incubated for 7 hours at 37°C in a humidified CO₂/air mixture (10%/90%). Vascular development is assessed by microscopy of fixed, whole-mounted embryos and sections.

Stimulation of coronary collateral growth can be measured in known animal models, including a rabbit model of peripheral limb ischemia and hind limb ischemia and a pig model of chronic myocardial ischemia (Ferrara et al., *Endocrine Reviews* 18:4-25, 1997). Test proteins are assayed in the presence and absence of VEGF and basic FGF to test for combinatorial effects. These models can be modified by the use of adenovirus or naked DNA for gene delivery as disclosed in more detail above, resulting in local expression of the test protein(s).

Angiogenic activity can also be tested in a rodent model of corneal neovascularization as disclosed by Muthukkaruppan and Auerbach, *Science* 205:1416-1418, 1979, wherein a test substance is inserted into a pocket in the cornea of an inbred mouse. For use in this assay, proteins are combined with a solid or semi-solid, biocompatible carrier, such as a polymer pellet. Angiogenesis is followed microscopically. Vascular growth into the corneal stroma can be detected in about 10 days.

Angiogenic activity can also be tested in the hamster cheek pouch assay (Höckel et al., *Arch. Surg.* 128:423-429, 1993). A test substance is injected subcutaneously into the cheek pouch, and after five days the pouch is examined under low magnification to determine the extent of neovascularization. Tissue sections can also be examined histologically.

Induction of vascular permeability is measured in assays designed to detect leakage of protein from the vasculature of a test animal (e.g., mouse or guinea pig) after administration of a test compound (Miles and Miles, *J. Physiol.* 118:228-257, 1952; Feng et al., *J. Exp. Med.* 183:1981-1986, 1996).

Wound-healing models include the linear skin incision model of Mustoe et al. (*Science* 237:1333, 1987). In a typical procedure, a 6-cm incision is made in the dorsal pelt of an adult rat, then closed with wound clips. Test substances and controls (in solution, gel, or powder form) are applied before primary closure. It is preferred to limit administration to a single application, although additional applications can be made on succeeding days by careful injection at several sites under the incision. Wound breaking strength is evaluated between 3 and 21 days post wounding. In a second model, multiple, small, full-thickness excisions are made on the ear of a rabbit. The cartilage in the ear splints the wound, removing the variable of wound contraction from the evaluation of closure. Experimental treatments and controls are applied. The geometry and anatomy of the wound site allow for reliable quantification of cell ingrowth and epithelial migration, as well as quantitative analysis of the biochemistry

pigs is evaluated (LeGrand et al., *Growth Factors* 8:307, 1993). Experimental treatments are applied daily on or under dressings. Seven days after wounding, granulation tissue thickness is determined. This model is preferred for dose-response

dermis are removed down to the panniculus carnosus in rodents or the subcutaneous fat in pigs. Experimental treatments are applied topically on or under a dressing, and can be applied daily if desired. The wound closes by a combination of contraction and

models are also known in the art (e.g., Cromack et al., *Surgery* **113**:36, 1993; Pierce et al., *Proc. Natl. Acad. Sci. USA* **86**:2229, 1989; Greenhalgh et al., *Amer. J. Pathol.* **136**:1235, 1990). Delay or prolongation of the wound healing process can be induced

are most commonly used as the experimental wound. Endpoints are as disclosed above for each type of wound. Subcutaneous implants can be used to assess compounds acting in the early stages of wound healing (Broadley et al., *Lab. Invest.* **61**:571, 1985;

polytetrafluoroethylene implants filled with bovine collagen) and placed subcutaneously in mice or rats. The interior of the implant is empty of cells, producing a "wound space" that is well-defined and separable from the preexisting tissue. This

Inhibition of tumor metastasis can be assessed in mice into which cancerous cells or tumor tissue have been introduced by implantation or injection (e.g., Brown, *Advan. Enzyme Regul.* 35:293-301, 1995; Conway et al., *Clin. Exp. Metastasis*

14:115-124, 1996).

Effects on fibrinolysis can be measured in a rat model wherein the enzyme batroxobin and radiolabeled fibrinogen¹ are administered to test animals. Inhibition of fibrinogen activation by a test compound is seen as a reduction in the

circulating level of the label as compared to animals not receiving the test compound. See, Lenfors and Gustafsson, *Semin. Thromb. Hemost.* 22:335-342, 1996.

The invention further provides polypeptides that comprise an epitope-bearing portion of a protein as shown in SEQ ID NO:M, wherein M is an even integer from 2 to 328. An "epitope" is a region of a protein to which an antibody can bind. See, for example, Geyssen et al., *Proc. Natl. Acad. Sci. USA* 81:3998-4002, 1984. Epitopes can be linear or conformational, the latter being composed of discontinuous regions of the protein that form an epitope upon folding of the protein. Linear epitopes are generally at least 6 amino acid residues in length. Relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for example, Sutcliffe et al., *Science* 219:660-666, 1983. Antibodies that recognize short, linear epitopes are particularly useful in analytic and diagnostic applications that employ denatured protein, such as Western blotting (Tobin, *Proc. Natl. Acad. Sci. USA* 76:4350-4356, 1979). Antibodies to short peptides may also recognize proteins in native conformation and will thus be useful for monitoring protein expression and protein isolation, and in detecting proteins in solution, such as by ELISA or in immunoprecipitation studies.

Antigenic, epitope-bearing polypeptides of the present invention are useful for raising antibodies, including monoclonal antibodies, that specifically bind to the corresponding protein. Antigenic, epitope-bearing polypeptides contain a sequence of at least six, preferably at least nine, more preferably from 15 to about 30 contiguous amino acid residues of a protein. Within certain embodiments of the invention, the polypeptides comprise 40, 50, 100, or more contiguous residues of a protein as shown in SEQ ID NO:M, up to the entire predicted mature protein or the primary translation product. It is preferred that the amino acid sequence of the epitope-bearing polypeptide is selected to provide substantial solubility in aqueous solvents, that is the sequence includes relatively hydrophilic residues, and hydrophobic residues are substantially avoided. Table 7 lists preferred hexapeptides for use as antigens. Within Table 7, each the amino termini of the hexapeptides are specified. Those skilled in the art will recognize that longer polypeptides comprising these hexapeptides can also be used and will often be preferred.

Table 7

Protein	Hexapeptide N-termini				
Z1055G2P	113	112	109	47	46
Z1183G7P	41	87	40	39	95
Z125G9P	72	70	19	18	68
Z128G9P	171	181	170	2	187
Z1297G2P	349	328	346	193	326
Z1345G3P	46	58	1	41	57
Z1345G5P	151	6	150	82	149
Z1659G4P	168	121	120	133	132
Z2007G2P	132	125	131	55	54
Z2263G5P	145	144	113	112	74
Z25829G3P	104	118	101	115	100
Z25999G1P	66	65	88	57	64
Z27842G1P	117	116	115	114	78
Z2812G3P	139	145	138	2	49
Z281G1P	100	99	18	92	98
Z2839G2P	68	169	6	67	4
Z287G9P	112	65	102	2	1
Z2887G7P	87	139	123	25	36
Z2972G5P	83	39	82	38	79
Z297G1P	105	104	103	2	62
Z3038G1P	87	84	103	50	100
Z321375G1P	6	49	5	60	59
Z3264G1P	139	138	70	137	69
Z3357G2P	115	107	114	99	113
Z4256G1P	317	169	222	57	310
Z4384G2P	223	69	41	222	82
Z446500G1P	38	63	37	46	62
Z4622G1P	71	21	62	20	52
Z4670G1P	43	62	2	42	13
Z555G20P	155	34	110	101	154
Z577G9P	121	120	145	144	104
Z700497G2P	73	64	89	43	88
Z700823G2P	107	73	12	35	57
Z700996G11P	24	31	78	30	39
Z701935G4P	90	2	33	96	89
Z702408G2P	91	80	26	79	90
Z74922G1P	54	36	53	119	52
Z790708G1P	214	149	41	212	201
Z791735G1P	49	48	91	2	90
Z792987G7P	85	208	207	84	121
Z793561G4P	56	51	49	48	34
Z794249G2P	388	380	463	199	38
Z799543G2P	145	144	97	143	38
Z807106G2P	1	121	51	47	80
Z807166G2P	118	61	28	71	27
Z807270G7P	56	195	194	163	55
Z807340G6P	6	78	5	39	4
Z807941G1P	115	70	113	112	69
Z809241G11P	82	80	131	79	36
Z809335G4P	680	664	679	339	163
Z809469G10P	146	145	89	34	116

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Table 7 cont'd

Z835361G1P	132	131	77	1	76
Z835510G5P	107	232	248	230	222
Z835548G2P	155	52	82	153	137
Z835840G8P	19	217	63	216	235
Z835892G6P	99	98	133	59	158
Z835944G6P	89	44	88	42	50
Z835999G1P	48	47	27	45	26
Z836222G4P	127	89	135	133	125
Z836250G2P	106	105	104	103	78
Z836307G1P	57	65	56	64	55
Z836325G9P	85	84	93	50	73
Z836355G5P	149	148	2	1	145
Z837892G2P	76	104	66	75	65
Z838027G3P	103	50	101	160	100
Z838054G2P	78	77	76	56	20
Z838468G2P	42	58	55	51	3
Z842039G5P	-----	1	24	23	22
Z842046G1P	60	51	50	22	48
Z846090G4P	25	24	86	35	23
Z846280G2P	143	28	39	140	38
Z846300G9P	106	68	123	104	30
Z846363G2P	117	116	115	207	114
Z846626G9P	48	65	121	47	46
Z846631G3P	131	130	51	106	76
Z849065G4P	206	205	159	203	213
Z849135G16P	2	1	210	50	209
Z849162G4P	63	30	62	39	29
Z849213G14P	30	79	56	25	78
Z849221G9P	201	122	200	199	163
Z849314G5P	113	124	123	135	133
Z849377G7P	134	152	53	92	52
Z849533G7P	88	127	126	87	59
Z849559G5P	34	60	32	2	124
Z849746G1P	172	171	170	42	41
Z849893G9P	41	91	27	24	65
Z855073G14P	36	50	123	35	122
Z855260G4P	147	42	41	76	75
Z869105G16P	24	22	71	20	70
Z869173G14P	88	87	24	86	22
Z869333G11P	81	36	77	76	74
Z869654G5P	36	34	51	2	32
Z869671G3P	288	54	287	239	121
Z869719G6P	168	102	101	142	100
Z873630G11P	135	134	47	133	44
Z873683G5P	113	112	111	62	45
Z873717G10P	77	76	40	75	39
Z873745G3P	1	8	112	127	125
Z873811G8P	83	40	78	38	144
Z873818G10P	43	42	39	111	149
Z874015G4P	86	85	254	245	84
Z874090G3P	37	35	43	34	77
Z874090G4P	180	21	90	19	2
Z874178G8P	45	112	59	58	57
Z874193G6P	26	225	24	224	50

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Table 7 cont'd

Z875409G1P	32	22	30	16	75
Z877091G9P	57	156	36	155	56
Z877300G8P	99	86	98	153	152
Z877532G7P	18	17	16	56	15
Z877574G3P	23	135	87	83	105
Z877975G4P	99	98	43	42	56
Z887014G7P	101	47	128	35	119
Z887031G1P	196	30	193	232	230
Z887042G3P	172	60	267	121	51
Z887280G5P	93	92	108	45	91
Z887300G2P	41	40	2	1	93
Z888627G9P	57	156	36	155	56
Z888644G7P	163	123	122	162	101
Z888879G3P	86	75	37	36	32
Z890990G20P	103	145	102	118	137
Z891399G5P	152	151	149	178	148
Z891519G8P	21	109	1	69	107
Z891532G2P	103	102	101	80	79
Z891575G17P	123	140	79	122	75
Z891639G1P	91	157	90	156	89
Z891682G2P	2	173	1	141	122
Z891886G1P	127	126	124	84	64
Z892025G7P	101	236	90	87	260
Z892026G1P	74	24	81	90	72
Z903906G4P	1	39	97	64	96
Z908203G11P	119	117	31	102	99
Z908225G14P	90	44	132	131	127
Z908460G2P	93	92	91	152	37
Z908463G2P	155	51	50	140	154
Z908593G16P	58	189	25	114	169
Z908644G15P	146	144	260	198	38
Z908655G7P	102	88	177	36	101
Z908668G10P	154	21	52	116	115
Z908874G13P	113	124	123	135	133
Z908957G1P	17	16	59	82	15
Z909165G15P	138	39	49	137	151
Z909204G1P	29	59	82	42	58
Z909337G10P	72	7	31	30	93
Z909460G4P	146	138	145	108	135
Z910197G6P	85	84	158	167	174
Z912187G1P	84	183	2	1	109
Z913549G1P	63	62	203	60	57
Z930005G2P	1	155	154	21	104
Z930582G14P	206	205	159	203	213
Z930757G12P	105	103	89	29	102
Z930798G6P	53	51	49	115	108
Z930857G2P	297	296	294	292	291
Z930898G2P	206	99	198	197	205
Z931019G10P	24	23	152	13	21
Z931276G1P	130	127	240	123	120
Z931363G4P	131	154	42	27	39
Z931654G1P	31	2	1	46	30
Z931828G2P	41	40	62	93	39
Z931874G4P	87	139	123	25	36

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Table 7 cont'd

Z935148G9P	191	190	79	166	58
Z935205G2P	107	82	105	68	67
Z935414G4P	273	247	224	140	272
Z935553G3P	2	175	118	1	174
Z935805G4P	216	213	202	56	201

As used herein, the term "antibodies" includes polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as F(ab')₂ and Fab fragments, single chain antibodies, and the like, including genetically engineered antibodies. Non-human antibodies can be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. One skilled in the art can generate humanized antibodies with specific and different constant domains (i.e., different Ig subclasses) to facilitate or inhibit various immune functions associated with particular antibody constant domains.

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to an immunogenic polypeptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of an immobilized or labeled polypeptide). Human antibodies can be produced in transgenic, non-human animals that have been engineered to contain human immunoglobulin genes as disclosed in WIPO Publication WO 98/24893. It is preferred that the endogenous immunoglobulin genes in these animals be inactivated or eliminated, such as by homologous recombination.

Antibodies are defined to be specifically binding if they bind to a target polypeptide with an affinity at least 10-fold greater than the binding affinity to control (non-target) polypeptide. It is preferred that the antibodies exhibit a binding affinity (K_d) of 10^6 M⁻¹ or greater, preferably 10^7 M⁻¹ or greater, more preferably 10^8 M⁻¹ or greater, and most preferably 10^9 M⁻¹ or greater. The affinity of a monoclonal antibody can be readily determined by one of ordinary skill in the art (see, for example, Scatchard, *Ann. NY Acad. Sci.* 51: 660-672, 1949).

Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see for example, Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications*, CRC Press, Inc., Boca Raton, FL, 1982). As

would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats. The immunogenicity of a polypeptide immunogen may be increased through the use of an adjuvant such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of a polypeptide of interest or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "haptene-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

A variety of assays known to those skilled in the art can be utilized to detect antibodies that specifically bind to a polypeptide of interest. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include concurrent immunoelectrophoresis, radio-immunoassays, radio-immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, Western blot assays, inhibition or competition assays, and sandwich assays.

Antibodies can be used, for example, to isolate target polypeptides by affinity purification, for diagnostic assays for determining circulating or localized levels of target polypeptides, for tissue typing, for cell sorting, for screening expression libraries; for generating anti-idiotypic antibodies, and as neutralizing antibodies or as antagonists to block protein activity *in vitro* and *in vivo*.

Additionally, antibodies generated to the MSP proteins described herein can be used, either individually or in combination, to monitor these proteins in bodily fluids. In particular, correlating changes in the level of expression of the MSP proteins with changes in metabolism, or progression of disease would be useful in studying metabolism disorders or monitoring the improvement or decline of diseases.

Polynucleotides and polypeptides of the present invention will additionally find use as educational tools as a laboratory practicum kits for courses related to genetics and molecular biology, protein chemistry and antibody production and analysis. Due to their unique polynucleotide and polypeptide sequences molecules of MSP can be used as standards or as "unknowns" for testing purposes. For example, MSP polynucleotides can be used as aids, such as, for example, to teach a student how to prepare expression constructs for bacterial, viral, and/or mammalian expression, including fusion constructs, wherein MSP are the genes to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides;

and other binding partners disclosed herein can be directly or indirectly conjugated to drugs, toxins, radionuclides, enzymes, enzyme substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles, and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Cytotoxic molecules, for example, can be directly or indirectly attached to the binding partner (e.g., by chemical coupling or as a fusion protein), and include bacterial or plant toxins (e.g., diphtheria toxin, *Pseudomonas* exotoxin, ricin, saporin, abrin, and the like); therapeutic radionuclides (e.g., iodine-131, rhenium-188 or yttrium-90) which can be directly attached to a polypeptide or antibody or indirectly attached through means of a chelating moiety; and cytotoxic drugs (e.g., adriamycin). Methods for preparing labeled reagents are known in the art. Within an alternative embodiment, the detectable signal or other function can be provided by a second member of a complement-anticomplement pair, which second member binds to the diagnostic reagent. For example, a first (unlabeled) antibody can be used to bind to a cell-surface polypeptide, after which a second, labeled antibody which binds to the first antibody is added. Other complement-anticomplement pairs are known in the art and include biotin/streptavidin.

Diagnostic reagents as disclosed herein can be used *in vivo* or *in vitro*. *In vitro* diagnostic assays include assays of tissue and fluid samples. Assays for protein in serum, for example, may be used to detect metabolic abnormalities characterized by over- or under-production of the protein, such as cancers, immune system abnormalities, infections, organ failure, metabolic imbalances, inborn errors of metabolism and other disease states. Proteins of the present invention can also be used in the detection of circulating autoantibodies, which are indicative of autoimmune disorders. Those skilled in the art will recognize that conditions related to protein underexpression or overexpression may be amenable to treatment by therapeutic manipulation of the relevant protein level(s). Proteins in serum can be quantitated by known methods known in the art, which include the use of antibodies in a variety of formats. Non-antibody binding partners, such as ligand-binding receptor fragments (commonly referred to as "soluble receptors") can also be used.

In general, diagnostic methods employing oligonucleotide probes or primers comprise the steps of (a) obtaining a genetic sample from a patient; (b) incubating the genetic sample with an oligonucleotide probe or primer as disclosed above, under conditions wherein the probe or primer will hybridize to a complementary polynucleotide sequence, to produce a first reaction product; and (c) comparing the first reaction product to a control reaction product. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient. Genetic samples for use within such methods include genomic DNA, cDNA,

- and RNA. Suitable assay methods in this regard include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, ligation chain reaction (Barany, *PCR Methods and Applications* 1:5-16, 1991), ribonuclease protection assays, and other genetic linkage analysis techniques known in the art (Sambrook et al., *ibid.*; Ausubel et. al., *ibid.*; A.J. Marian, *Chest* 108:255-65, 1995). Ribonuclease protection assays (see, e.g., Ausubel et al., *ibid.*, ch. 4) comprise the hybridization of an RNA probe to a patient RNA sample, after which the reaction product (RNA-RNA hybrid) is exposed to RNase. Hybridized regions of the RNA are protected from digestion. Within PCR assays, a patient genetic sample is incubated with a pair of oligonucleotide primers, and the region between the primers is amplified and recovered. Changes in size, amount, or sequence of recovered product are indicative of mutations in the patient. Another PCR-based technique that can be employed is single strand conformational polymorphism (SSCP) analysis (Hayashi, *PCR Methods and Applications* 1:34-38, 1991).). Chromosomal localization data can be used to correlate AFP gene locations with known genetic disorders using, for example, the OMIM™ Database, Johns Hopkins University, 2000 (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>).

Table 8 show the chromosomal localization of the polynucleotide sequences encoding some of the polypeptides described herein.

TABLE 8	
Protein	Chromosomal Localization
Z125G9P	17p12
Z281G1P	22
Z287G9P	18q22.1
Z577G9P	4p15.1
Z1055G2P	yp11.2
Z1183G7P	12q
Z1345G3P	22q12.2
Z1659G4P	6q26-q27
Z2007G2P	7q21.3
Z2972G5P	3q27.3
Z3038G1P	17q12
Z3264G1P	8p11.23
Z3357G2P	7q31.1
Z25999G1P	22
Z700497G2P	11q22.1
Z700823G2P	Xq24
Z702408G2P	19
Z793561G4P	16q24.1
Z807166G2P	16q12.1
Z809335G4P	15q15.3
Z835892G6P	14
Z835999G1P	15q22.32
Z836222G4P	6q15-16.3
Z836325G9P	18q23
Z836355G5P	20q13.2-13.33
Z837892G2P	14
Z838468G2P	8q22.3
Z842039G5P	10q21.3
Z842046G1P	4
Z846090G4P	2q32.2
Z846280G2P	8q12.1
Z846631G3P	1p36.11-36.23
Z846626G9P	8q24.3
Z849135G16P	20
Z849213G14P	12q23.3
Z849559G5P	10q26.3
Z849746G1P	11q24.3
Z855073G14P	15q15
Z855260G4P	2q34
Z869105G16P	6q26
Z869173G14P	5p15.32
Z869654G5P	5q11.2

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TABLE 8 cont.	
Protein	Chromosomal Localization
Z869719G6P	6q14.3-16.1
Z873683G5P	5q14.3
Z873745G3P	6q27
Z874015G4P	18p11.32
Z874090G3P	8p21.3
Z874090G4P	8p21.3
Z874178G8P	11p12
Z874193G6P	6q21
Z877091G9P	7q36.3
Z877532G7P	5p14.1
Z877975G4P	15q11.2
Z887014G7P	8p23.3
Z887280G5P	14q24.1
Z888627G9P	7q36.3
Z888644G7P	15q15.3
Z890990G20P	3q26.2
Z891399G5P	16q22.2
Z891575G17P	17p12
Z891682G2P	4q31.1
Z892025G7P	8q21.3
Z892026G1P	10q22.1
Z908225G14P	Xq26.3-27.3
Z908460G2P	14
Z908593G16P	10p15.1
Z908644G15P	1p22.2-31.1
Z909165G15P	13q13.3
Z909204G1P	9p24.2
Z909460G4P	5q15
Z930798G6P	20q12
Z930898G2P	20
Z931019G10P	18q11.2
Z931363G4P	17
Z935148G9P	17p11.2
Z935205G2P	3q26.1
Z935553G3P	Xp22.32

Polynucleotides of the present invention, including fragments thereof,

5 can also be used for radiation hybrid mapping, a somatic cell genetic technique

developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox et al., *Science* 250:245-50, 1990). Partial or full knowledge of a gene's sequence allows the design of PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Commercially available radiation hybrid mapping panels which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL), are available. These panels enable rapid, PCR-based chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other nonpolymorphic and polymorphic markers within a region of interest, allowing the establishment of directly proportional physical distances between newly discovered genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful for a number of purposes, including: 1) determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms, such as YACs, BACs or cDNA clones; 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region; and 3) cross-referencing model organisms, such as mouse, which may aid in determining what function a particular gene might have.

If a mammal has an insufficiency of a protein of interest (due to, for example, a mutated or absent gene), the corresponding wild-type gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a protein of interest is introduced into the animal using a viral vector. Such vectors include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes simplex virus 1 (HSV1) vector (Kaplit et al., *Molec. Cell. Neurosci.* 2:320-30, 1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. (*J. Clin. Invest.* 90:626-30, 1992); and a defective adeno-associated virus vector (Samulski et al., *J. Virol.* 61:3096-101, 1987; Samulski et al., *J. Virol.* 63:3822-28, 1989).

Within another embodiment, a gene of interest is introduced into an animal by liposome-mediated transfection ("lipofection") essentially as disclosed above. Lipofection can be used to introduce exogenous genes into specific organs.

In another embodiment, polypeptide-toxin fusion proteins or antibody/fragment-toxin fusion proteins may be used for targeted cell or tissue inhibition or ablation, such as in cancer therapy. Of particular interest in this regard are conjugates of an MSP protein and a cytotoxin, which can be used to target the cytotoxin to a tumor or other tissue that is undergoing undesired angiogenesis or neovascularization.

In another embodiment, polypeptide-toxin fusion proteins or other binding partner-linked toxins may be used for targeted cell or tissue inhibition or ablation (for instance, to treat cancer cells or tissues). Target cells (i.e., those displaying a receptor for a polypeptide of interest) bind the polypeptide-toxin conjugate, which is then internalized, killing the cell. The effects of receptor-specific cell killing (target ablation) are revealed by changes in whole animal physiology or through histological examination. Thus, ligand-dependent, receptor-directed cytotoxicity can be used to enhance understanding of the physiological significance of a protein ligand. A preferred such toxin is saporin. Mammalian cells have no receptor for saporin, which is non-toxic when it remains extracellular. Alternatively, if the polypeptide of interest has multiple functional domains (i.e., an activation domain or a ligand binding domain, plus a targeting domain), a fusion protein including only the targeting domain may be suitable for directing a detectable molecule, a cytotoxic molecule or a complementary molecule to a cell or tissue type of interest. In instances where the domain-only fusion protein includes a complementary molecule, the anti-complementary molecule can be conjugated to a detectable or cytotoxic molecule. Such domain-complementary molecule fusion proteins thus represent a generic

targeting vehicle for cell- or tissue-specific delivery of generic anti-complementary-detectable/cytotoxic molecule conjugates.

The bioactive conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action.

For pharmaceutical use, the proteins of the present invention are formulated according to conventional methods. Routes of delivery include topical, mucosal, and parenteral, the latter including intravenous and subcutaneous delivery. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a protein of the present invention in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, diluents, fillers, emulsifiers, preservatives, solubilizers, buffering agents, wetting agents, stabilizers, colorings, penetration enhancers, albumin to prevent protein loss on vial surfaces, etc. Topical formulations are typically provided as liquids, ointments, salves, gels, emulsions and the like. Methods of formulation are well known in the art and are disclosed, for example, in *Remington: The Science and Practice of Pharmacy*, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995. Therapeutic doses will be determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Proteins of the present invention will generally be formulated to provide a dose of from 0.01 μg to 100 mg per kg patient weight per day, more commonly from 0.1 μg to 10 mg/kg/day, still more commonly from 0.1 μg to 1.0 mg/kg/day. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years. In general, a therapeutically effective amount is an amount sufficient to produce a clinically significant change in the targeted condition.

Within the laboratory research field, the proteins of the present invention can be used as molecular weight standards, or as standards in the analysis of cell phenotype, and as reagents for the study of cells, receptors, and other binding molecules. Such reagents will generally further comprise a second moiety, such as a label, binding partner, or toxin, that facilitates the detection of the protein when bound to its target. Many such systems are known in the art and are summarized above. Receptors and other cell-surface binding sites for proteins of the present invention can be identified by exposing a population of cells to a labeled protein under physiologic conditions, whereby the protein binds to the surface of the cell. Cells bearing receptors

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for a protein of interest can also be identified using the protein joined to a toxin, whereby receptor-bearing cells are killed by the toxin.

MSP proteins and antagonists thereof can be used as standards in assays of protein and protein inhibitors in both clinical and research settings. Such assays can comprise any of a number of standard formats, include radioreceptor assays and ELISAs. Protein standards can be prepared in labeled form using a radioisotope, enzyme, fluorophore, or other compound that produces a detectable signal. The proteins can be packaged in kit form, such kits comprising one or more vials containing the MSP protein and, optionally, a diluent, an antibody, a labeled binding protein, etc. Assay kits can be used in the research laboratory to detect protein and inhibitor activities produced by cultured cells or test animals.

Proteins of the present invention may also be used as protein and amino acid supplements, including hydrolysates. Specific uses in this regard include use as animal feed supplements and as cell culture components. Proteins rich in a particular amino acid can be used as a source of that amino acid.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1

A protein of the present invention ("MSP") is produced in *E. coli* using a His₆ tag/maltose binding protein (MBP) double affinity fusion system as generally disclosed by Pryor and Leiting, *Prot. Expr. Pur.* 10:309-319, 1997. A thrombin cleavage site is placed at the junction between the affinity tag and MSP sequences.

The fusion construct is assembled in the vector pTAP98, which comprises sequences for replication and selection in *E. coli* and yeast, the *E. coli* tac promoter, and a unique SmaI site just downstream of the MBP-His₆-thrombin site coding sequences. The MSP cDNA is amplified by PCR using primers each comprising 40 bp of sequence homologous to vector sequence and 25 bp of sequence that anneals to the cDNA. The reaction is run using Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) for 30 cycles of 94°C, 30 seconds; 60°C, 60 seconds; and 72°C, 60 seconds. One microgram of the resulting fragment is mixed with 100 ng of SmaI-cut pTAP98, and the mixture is transformed into yeast to assemble the vector by homologous recombination (Oldenburg et al., *Nucl. Acids. Res.* 25:451-452, 1997). Ura⁺ transformants are selected.

Plasmid DNA is prepared from yeast transformants and transformed into *E. coli* MC1061. Pooled plasmid DNA is then prepared from the MC1061

transformants by the miniprep method after scraping an entire plate. Plasmid DNA is analyzed by restriction digestion.

E. coli strain BL21 is used for expression of MSP. Cells are transformed by electroporation and grown on minimal glucose plates containing casamino acids and ampicillin.

Protein expression is analyzed by gel electrophoresis. Cells are grown in liquid glucose media containing casamino acids and ampicillin. After one hour at 37°C, IPTG is added to a final concentration of 1mM, and the cells are grown for an additional 2-3 hours at 37°C. Cells are disrupted using glass beads, and extracts are prepared.

Example 2

Larger scale cultures of MSP transformants are prepared by the method of Pryor and Leiting (*ibid.*). 100-ml cultures in minimal glucose media containing casamino acids and 100 µg/ml ampicillin are grown at 37°C in 500-ml baffled flasks to OD₆₀₀ ≈ 0.5. Cells are harvested by centrifugation and resuspended in 100 ml of the same media at room temperature. After 15 minutes, IPTG is added to 0.5 mM, and cultures are incubated at room temperature (ca. 22.5°C) for 16 to 20 hours with shaking at 125 rpm. The culture is harvested by centrifugation, and cell pellets are stored at -70°C.

Example 3

For larger-scale protein preparation, 500-ml cultures of *E. coli* BL21 expressing the MSP-MBP-His₆ fusion protein are prepared essentially as disclosed in Example 2. Cell pellets are resuspended in 100 ml of binding buffer (20 mM Tris, pH 7.58, 100 mM NaCl, 20 mM NaH₂PO₄, 0.4 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride [Pefabloc® SC; Boehringer-Mannheim], 2 µg/ml Leupeptin, 2 µg/ml Aprotinin). The cells are lysed in a French press at 30,000 psi, and the lysate is centrifuged at 18,000 x g for 45 minutes at 4°C to clarify it. Protein concentration is estimated by gel electrophoresis with a BSA standard.

Recombinant MSP fusion protein is purified from the lysate by affinity chromatography. Immobilized cobalt resin (Talon® resin; Clontech Laboratories, Inc., Palo Alto, CA) is equilibrated in binding buffer. One ml of packed resin per 50 mg protein is combined with the clarified supernatant in a tube, and the tube is capped and sealed, then placed on a rocker overnight at 4°C. The resin is then pelleted by centrifugation at 4°C and washed three times with binding buffer. Protein is eluted with binding buffer containing 0.2 M imidazole. The resin and elution buffer are

mixed for at least one hour at 4°C, the resin is pelleted, and the supernatant is removed. An aliquot is analyzed by gel electrophoresis, and concentration is estimated. Amylose resin is equilibrated in amylose binding buffer (20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 10 mM EDTA) and combined with the supernatant from the Talon resin at a ratio of 2 mg fusion protein per ml of resin. Binding and washing steps are carried out as disclosed above. Protein is eluted with amylose binding buffer containing 10 mM maltose using as small a volume as possible to minimize the need for subsequent concentration. The eluted protein is analyzed by gel electrophoresis and staining with Coomassie blue using a BSA standard, and by Western blotting using an anti-MBP antibody.

Example 4

An expression plasmid containing all or part of a polynucleotide encoding MSP is constructed via homologous recombination. An MSP coding sequence comprising the ORF with 5' and 3' ends corresponding to the vector sequences flanking the insertion point is prepared by PCR. The primers for PCR each include from 5' to 3' end: 40 bp of flanking sequence from the vector and 17 bp corresponding to the amino or carboxyl termini from the open reading frame of MSP.

Ten μ l of the 100 μ l PCR reaction mixture is run on a 0.8% low-melting-temperature agarose (SeaPlaque GTG®; FMC BioProducts, Rockland, ME) gel with 1 x TBE buffer for analysis. The remaining 90 μ l of the reaction mixture is precipitated with the addition of 5 μ l 1 M NaCl and 250 μ l of absolute ethanol. The plasmid pZMP6, which has been cut with SmaI, is used for recombination with the PCR fragment. Plasmid pZMP6 is a mammalian expression vector containing an expression cassette having the cytomegalovirus immediate early promoter, multiple restriction sites for insertion of coding sequences, a stop codon, and a human growth hormone terminator; an *E. coli* origin of replication; a mammalian selectable marker expression unit comprising an SV40 promoter, enhancer and origin of replication, a DHFR gene, and the SV40 terminator; and URA3 and CEN-ARS sequences required for selection and replication in *S. cerevisiae*. It was constructed from pZP9 (deposited at the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, under Accession No. 98668) with the yeast genetic elements taken from pRS316 (available from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA, under Accession No. 77145), an internal ribosome entry site (IRES) element from poliovirus, and the extracellular domain of CD8 truncated at the C-terminal end of the transmembrane domain.

- One hundred microliters of competent yeast (*S. cerevisiae*) cells are independently combined with 10 μ l of the various DNA mixtures from above and transferred to a 0.2-cm electroporation cuvette. The yeast/DNA mixtures are electropulsed using power supply (BioRad Laboratories, Hercules, CA) settings of 0.75 kV (5 kV/cm), ∞ ohms, 25 μ F. To each cuvette is added 600 μ l of 1.2 M sorbitol, and the yeast is plated in two 300- μ l aliquots onto two URA-D plates (1.8% agar in 2% D-glucose, 0.67% yeast nitrogen base without amino acids, 0.056% -Ura -Trp -Thr powder [made by combining 4.0 g L-adenine, 3.0 g L-arginine, 5.0 g L-aspartic acid, 2.0 g L-histidine, 6.0 g L-isoleucine, 8.0 g L-leucine, 4.0 g L-lysine, 2.0 g L-methionine, 6.0 g L-phenylalanine, 5.0 g L-serine, 5.0 g L-tyrosine, and 6.0 g L-valine], and 0.5% 200X tryptophan, threonine solution [3.0% L-threonine, 0.8% L-tryptophan in H₂O]) and incubated at 30°C. After about 48 hours, the Ura⁺ yeast transformants from a single plate are resuspended in 1 ml H₂O and spun briefly to pellet the yeast cells. The cell pellet is resuspended in 1 ml of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA). Five hundred microliters of the lysis mixture is added to an Eppendorf tube containing 300 μ l acid-washed glass beads and 200 μ l phenol-chloroform, vortexed for 1 minute intervals two or three times, and spun for 5 minutes in an Eppendorf centrifuge at maximum speed. Three hundred microliters of the aqueous phase is transferred to a fresh tube, and the DNA is precipitated with 600 μ l ethanol (EtOH), followed by centrifugation for 10 minutes at 4°C. The DNA pellet is resuspended in 10 μ l H₂O.

- Transformation of electrocompetent *E. coli* host cells (Electromax DH10B™ cells; obtained from Life Technologies, Inc., Gaithersburg, MD) is done with 0.5-2 ml yeast DNA prep and 40 μ l of cells. The cells are electropulsed at 1.7 kV, 25 μ F, and 400 ohms. Following electroporation, 1 ml SOC (2% Bacto™ Tryptone (Difco, Detroit, MI), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) is plated in 250- μ l aliquots on four LB AMP plates (LB broth (Lennox), 1.8% Bacto™ Agar (Difco), 100 mg/L Ampicillin).

- Individual clones harboring the correct expression construct for MSP are identified by restriction digest to verify the presence of the MSP insert and to confirm that the various DNA sequences have been joined correctly to one another. The inserts of positive clones are subjected to sequence analysis. Larger scale plasmid DNA is isolated using a commercially available kit (QIAGEN Plasmid Maxi Kit, Qiagen, Valencia, CA) according to manufacturer's instructions. The correct construct is designated pZMP6/MSP.

Recombinant protein is produced in BHK cells transfected with pZMP6/MSP. BHK 570 cells (ATCC CRL-10314) are plated in 10-cm tissue culture

- dishes and allowed to grow to approximately 50 to 70% confluence overnight at 37°C, 5% CO₂, in DMEM/FBS media (DMEM, Gibco/BRL High Glucose; Life Technologies), 5% fetal bovine serum (Hyclone, Logan, UT), 1 mM L-glutamine (JRH Biosciences, Lenexa, KS), 1 mM sodium pyruvate (Life Technologies). The cells are then transfected with pZMP6/MSP by liposome-mediated transfection using a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium-trifluoroacetate and the neutral lipid dioleoyl phosphatidylethanolamine in membrane-filtered water (Lipofectamine™ Reagent; Life Technologies, Gaithersburg, MD), in serum free (SF) media (DMEM supplemented with 10 mg/ml transferrin, 5 mg/ml insulin, 2 mg/ml fetuin, 1% L-glutamine and 1% sodium pyruvate). The plasmid is diluted into 15-ml tubes to a total final volume of 640 µl with SF media. 35 µl of the lipid mixture is mixed with 605 µl of SF medium, and the resulting mixture is allowed to incubate approximately 30 minutes at room temperature. Five milliliters of SF media is then added to the DNA:lipid mixture. The cells are rinsed once with 5 ml of SF media, aspirated, and the DNA:lipid mixture is added. The cells are incubated at 37°C for five hours, then 6.4 ml of DMEM/10% FBS, 1% PSN media is added to each plate. The plates are incubated at 37°C overnight, and the DNA:lipid mixture is replaced with fresh 5% FBS/DMEM media the next day. On day 5 post-transfection, the cells are split into T-162 flasks in selection medium (DMEM + 5% FBS, 1% L-Gln, 1% NaPyr, 1 µM methotrexate). Approximately 10 days post-transfection, two 150-mm culture dishes of methotrexate-resistant colonies from each transfection are trypsinized, and the cells are pooled and plated into a T-162 flask and transferred to large-scale culture.

- From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.